environmental microbiology

Environmental Microbiology (2014) doi:10.1111/1462-2920.12385

# The LonB protease controls membrane lipids composition and is essential for viability in the extremophilic haloarchaeon *Haloferax volcanii*

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## Summary

**Although homologs of the ATP-dependent Lon protease exist in all domains of life, the relevance of this protease in archaeal physiology remains a mystery. In this study, we have constructed and phenotypically characterized deletion and conditional *lon* mutants in the model haloarchaeon *Haloferax volcanii* to elucidate the role of the unusual membrane-bound LonB protease in archaea. *Hvlon* could be deleted from the chromosome only when a copy of the *wild type* gene was provided *in trans* suggesting that Lon is essential for survival in this archaeon. Successful complementation of the lethal phenotype of Δ*Hvlon* was attained by expression of the heterologous protease gene *Nmlon* from the haloalkaliphilic archaeon *Natrialba magadii*, meaning that the biological function of Lon is conserved in these organisms. Suboptimal cellular levels of Lon protein affected growth rate, cell shape, cell pigmentation, lipid composition and sensitivity to various antibiotics. The contents of bacterioruberins and some polar lipids were increased in the *lon* mutants suggesting that Lon is linked to maintenance of membrane lipid balance which likely affects cell viability in this archaeon. The phenotypes associated to a membrane-bound LonB protease mutant were examined for the first time providing insight on the relevance of this protease in archaeal physiology.**

## Introduction

Proteolysis is a key process in cell physiology. ATP-dependent proteases are fundamental for protein quality control as well as for the regulation of physiological processes controlling the level of key proteins/enzymes. These events allow the cells to adapt to environmental and developmental challenges. Various types of energy-dependent proteases which belong to the AAA+ protein family (ATPases associated with various cellular activities) have been characterized, including: proteasomes, HslUV, Clp, FtsH and the Lon protease (Sauer and Baker, 2011). These enzymes are organized as compartmentalized structures consisting of the AAA+ domain (responsible for recognition, binding and unfolding of protein substrates) and the proteolytic chamber. These two compartments are assembled from independent polypeptides (proteasomes, Clp, HslUV) or can be fused into a single protein (FtsH and Lon). In addition to their relevance in cell physiology, energy-dependent proteases are attractive for research as they are implicated in bacterial infections and many human diseases (Butler *et al*., 2006; Tatsuta and Langer, 2008).

Archaea have been classified as a separate domain from Bacteria and Eukarya due to their evolutionary origin and distinct molecular features (Woese *et al*., 1990). Although archaea have been isolated from various types of habitats (Robertson *et al*., 2005), most archaeal species thrive in extreme environments, which means that they have evolved to adapt their molecules and/or cellular processes to one or various harsh conditions. Compared with the other two domains, less is known on archaeal physiology, including proteolysis. Even though protein breakdown affects all life forms, the importance of this process in the 'extremophilic' lifestyle is not clear. Archaeal genomes encode homologs of various protease families which occur in other organisms, nevertheless, the biological role of many of these enzymes has not been addressed in these unusual microbes. Only two ATP-dependent proteases have been identified in archaea: the 20S proteasome-PAN system and the Lon protease. Archaeal proteasomes have been characterized from methanogens, thermophiles and extreme halophiles (Maupin-Furlow *et al*., 2005). In haloachaea, such as *Haloferax volcanii*, proteasomes are required for cell growth and stress responses, and total depletion of proteasomes rendered the cells unviable (Zhou *et al*., 2008).

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Lon proteases are present in all domains of life including Bacteria, Archaea and Eukarya (Maupin-Furlow *et al*., 2005). Based on their structure, they have been classified in two groups: LonA and LonB (Rotanova *et al*., 2004). The LonA subfamily occurs in bacteria and eukaryotic organelles while LonB is found almost exclusively in archaea, with the exception of a few LonB-like proteins that are present in some bacteria. LonA is encoded by a single gene and contains an amino-terminal domain (LAN), a central ATPase (AAA+) domain and a C-terminal protease domain (P-domain). LonB enzymes lack the LAN domain but contain a hydrophobic segment in the ATPase domain that anchors the protein into the cytoplasmic membrane.

The structure, biochemistry and mechanism of protein degradation as well as the biological functions of LonA proteases have been extensively examined in *Escherichia coli* (and other bacteria) and in eukaryotic cells (Ngo and Davies, 2007; Van Melderen and Aertsen, 2009). With a few exceptions, Lon is not essential for cell viability. However, in some bacterial species including *E. coli, lon* mutants show several defects such as accumulation of abnormal proteins, mucoid colonies, sensitivity to DNA damaging agents and defects in cell division resulting in the formation of long filaments (reviewed in Van Melderen and Aertsen, 2009). Lon is a key enzyme in the adaptation of bacteria to various stress conditions and controls a number of cellular functions including plasmid maintenance, cell cycle and differentiation, sporulation, motility and biofilm formation, antibiotic resistance and pathogenicity (reviewed in Tsilibaris *et al*., 2006; Breidenstein and Hancock, 2013).

Relatively little is known on the biology of LonB proteases. Previous studies have examined the biochemical and catalytic properties as well as the crystal structures of the Protease and ATPase domains of the LonB enzymes within thermophilic and methanogen representatives of archaea (Fukui *et al*., 2002; Besche *et al*., 2004; Im *et al*., 2004; Botos *et al*., 2005; Cha *et al*., 2010). However, the physiological significance of this membrane-bound Lon homolog has not been investigated in any archaeon so far.

Aiming to understand proteolysis in the context of the halophilic group of archaea, we previously cloned the gene and characterized the recombinant LonB protease of the haloalkaliphilic archaeon *Natrialba magadii* (NmLon) (Sastre *et al*., 2010). This study confirmed the localization of this enzyme in the cytoplasmic membrane and revealed that the archaeal-type LonB binds to DNA, just like the LonA homologs (Lee and Suzuki, 2008). In many haloarchaea, including *N. magadii* and *H. volcanii*, there is a single copy of the *lon* gene located upstream of a sequence encoding a hypothetical protein annotated as 'abortive infection protein' (Abi) related to the CAAX prenyl endopeptidase family. We demonstrated that these genes are linked at the transcript level in *N. magadii* (Sastre *et al*., 2010).

To get insight on the role of the LonB homolog in archaeal physiology, deletion and conditional mutants of the *lon* gene were constructed and phenotypically characterized in the model haloarchaeon *H. volcanii*. We show that LonB is essential for cell growth and viability in this archaeon. *H volcanii* mutants with suboptimal amounts of Lon protein showed increased concentrations of the polyisoprenoid compounds (bacterioruberins and polar lipids), indicating that LonB is required to maintain the proper lipid composition of cell membranes in *H. volcanii*.

## Results

### *Lon mRNA expression changes under different growth conditions in* H. volcanii

To explore the role of the Lon protease in *H. volcanii*, the relative contents of Lon mRNA were analysed during growth under various conditions: nutrient limitation, hypo and hyper salinity and UV light irradiation.

*Haloferax volcanii* cells were grown in rich medium to mid exponential phase and then transferred to fresh MGM or Hv-Min media and further incubated for several hours. The cells growing in Hv-Min showed a lower growth rate ($\mu$) than those in MGM medium ($0.09 \pm 0.008$ and $0.259 \pm 0.03$ $h^{-1}$ respectively). While Lon transcripts were detected under both culture conditions, the relative content of Lon messenger RNA (mRNA) after 3 h of the shift was higher in cells growing in Hv-Min (5-fold) compared with those in MGM (Fig. 1A). This result suggests that Lon is required for the adaptation and/or survival of *H. volcanii* under stringent nutritional conditions, probably to degrade endogenous protein resources to supply amino acids for biosynthesis.

*Haloferax volcanii* is a moderate halophile and can grow at various salt concentrations. To investigate the effect of medium salinity on Lon expression, *H. volcanii* cultures growing in optimal conditions (2.5 M NaCl) were diluted with fresh medium such that the final NaCl concentrations were 1.25, 2.5 and 3.8 M, and the cultures were further incubated. Growth rate and cell yield were fairly similar in 2.5 and 3.8 M NaCl ($0.078 \pm 0.007$ and $0.057 \pm 0.001$ $h^{-1}$; $OD_{600} \sim 1.5$), while in 1.25 M the cultures decreased growth rate ($0.043 \pm 0.004$ $h^{-1}$) and arrested growth within $OD_{600} \sim 0.5$. The cellular content of Lon mRNA varied in direct correlation with medium osmolarity, being the lowest under hyposalinity and the highest in 3.8 M NaCl (~ 2-fold relative to 2.5 M respectively) (Fig. 1B). This observation suggests a link between Lon and the mechanism/s of osmotic adaptation, being particularly important at high concentrations.

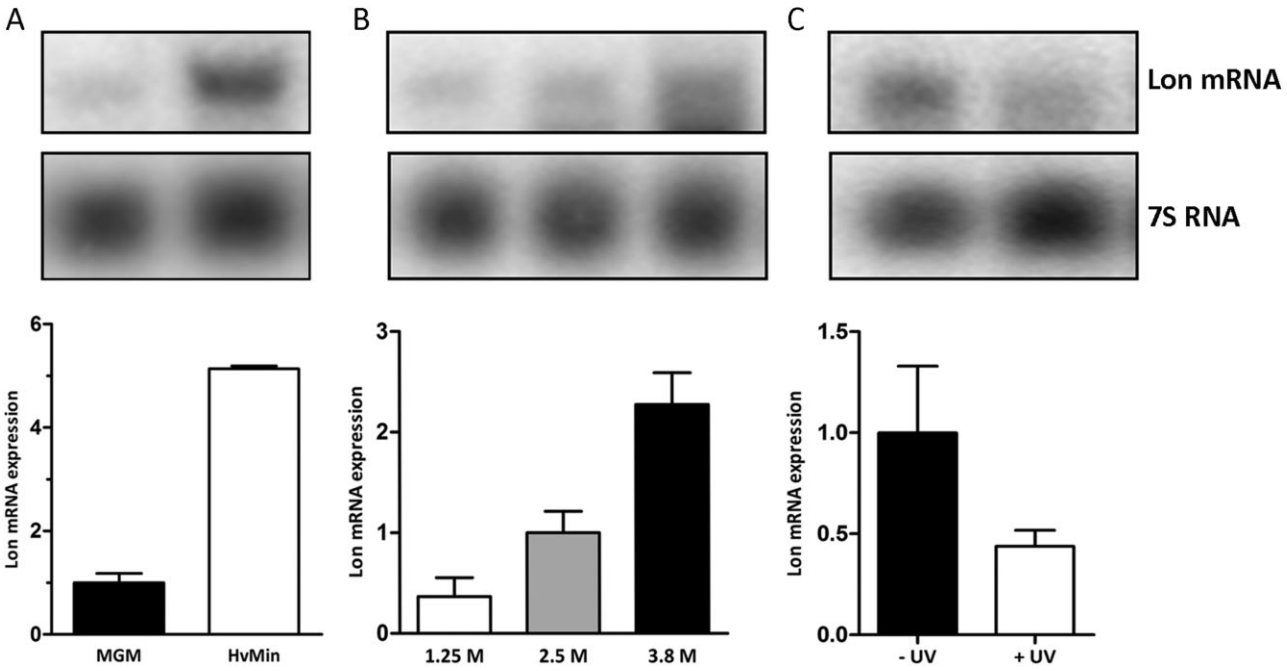


**Fig. 1.** Expression of Lon mRNA in *H. volcanii* cultures subjected to stress conditions.
A. *Haloferax volcanii* cells growing in MGM medium were pelleted and suspended in MGM or Hv-Min media.
B. Cultures of *H. volcanii* growing in MGM medium (2.5 M NaCl) were diluted with fresh medium to low (1.25 M), optimal (2.5 M) or high (3.8 M) salinity. The cultures in A and B were incubated at 42°C and sampled.
C. *Haloferax volcanii* cells were irradiated with UV-B light for 8 min and left to recuperate for 1 h. Lon mRNA expression was analysed by Northern blotting, and the cellular concentration of *lon* transcripts was normalized to that of the constitutively expressed 7S RNA. Values are relative to those obtained in MGM (A), 2.5 M NaCl (B) or in absence of UV light (C). The results are representative of at least three independent experiments.

To examine the effect of UV light, *H. volcanii* cells were irradiated with UV-B light for 8 min and left to recuperate for 1 h. We had previously observed that these conditions induced a lag period in *H. volcanii* H26 after which the cells resumed growth. Fig. 1C shows that the relative expression of Lon transcripts decreased by 2-fold after UV irradiation, suggesting that Lon may have a negative effect on the response of *H. volcanii* to UV light stress.

Although variations at the mRNA level were consistent and reproducible in different experiments, the cellular content of Lon protein, as estimated by Western blotting with anti-NmLon antibodies, did not evidence detectable changes during the stress conditions tested (not shown).

### *Lon is an essential protease in* H. volcanii

To address the physiological relevance of Lon, a genetic approach was used to generate a deletion mutant strain of *H. volcanii* in *Hvlon* (HVO_0783) (Allers *et al.*, 2004) and *H. volcanii* H26 (Δ*pyrE*) as the parent strain. Colonies of *H. volcanii* that grew on selective medium (Hv-min + FOA containing 10 μg $ml^{-1}$ uracil) were screened by Dot blotting with a specific probe for *Hvlon* and/or by polymerase chain reaction (PCR) using primers internal to the *lon* sequence (Supporting Information Table S1). Cells that had deleted the *lon* gene could not be detected out of ~ 70 clones examined from three independent experiments suggesting that Lon was critical for cell viability. To examine this possibility, the pop-in pop-out method was then applied to *H. volcanii* cells harbouring the expression plasmid pRV-*Nmlon* encoding the heterologous Lon protease from the haloalkaliphilic archaeon *N. magadii* (NmLon), which was available in our laboratory (Sastre *et al.*, 2010). NmLon is 85% similar to the *H. volcanii* protease (HvLon) at the protein level although it has a slightly higher molecular mass (84 kDa vs 75 kDa respectively). Using this approach, several clones (3 out of 12 clones analysed) were detected which had deleted the endogenous *lon* gene. Loss of this gene was validated by PCR using primers hybridizing to the *Hvlon* sequence (expected product 2.08 kbp) and external to the 5′ and 3′ flanking regions (expected amplicon 4 kbp) in the mutant colonies (Fig. 2A and B respectively). To verify that NmLon was expressed in the Δ*Hvlon*/p-*Nmlon* mutant (denoted as HVLON1) and to corroborate the absence of the endogenous protease, one clone was selected and analysed by Western blotting with anti-NmLon polyclonal antibodies (Fig. 2C). Note the difference in electrophoretic mobility between NmLon and the endogenous HvLon

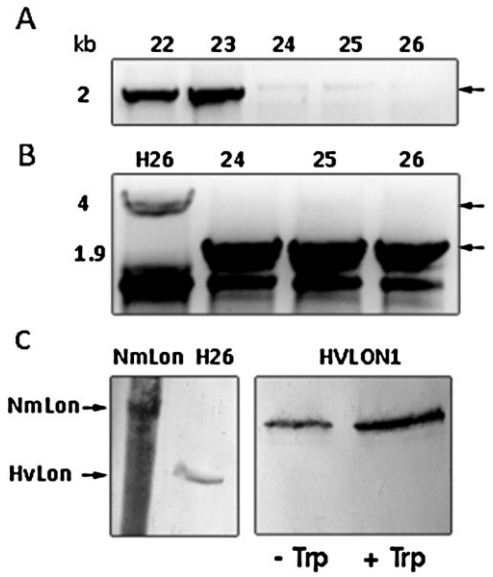


**Fig. 2.** Verification of the mutant strain HVLON1. Deletion of the endogenous *lon* gen in *H. volcanii* H26 harbouring the expression plasmid pRV-*Nmlon* was confirmed by locus-specific PCR using primer pairs (Table 1) designed to anneal within (A) or outside (B) the *lon* gene. Numbers 22–26 correspond to the different clones examined. Expected amplicon sizes are indicated by arrows.
C. Western blot of HVLON1 cell lysates (induced or not with 2 mM Trp) probed with anti-NmLon antibodies. NmLon: purified recombinant Lon protease from *N. magadii* (positive control).

expressed in the parent strain H26 (left panel). While no signals corresponding to HvLon were detected in the HVLON1 mutant strain, a polypeptide immunoreactive with anti-NmLon antibodies (expressed under the control of the P*tnaA* promoter) was observed, whose concentration increased upon induction with Trp (right panel).

The growth rates of HVLON1 and parent strains were compared in Hv-Min liquid medium with or without Trp (2 mM). In the absence of Trp, both strains grew similarly, having HVLON1 a moderately lower growth rate than H26 *wt* ($0.075 \pm 0.004$ and $0.126 \pm 0.012$ $h^{-1}$ respectively). This means that the basal amount of the heterologous protease synthesized from the plasmid allowed survival and growth of the mutant cells lacking the endogenous enzyme. Addition of Trp to the cultures significantly affected growth of HVLON1 ($0.042 \pm 0.003$ $h^{-1}$) probably due to overexpression of NmLon.

Altogether, these results evidence that the Lon protease is needed to maintain cell viability in *H. volcanii,* at least under the conditions used in this study, and showed that the function of this enzyme is conserved between these two haloarchaea.

Cells of HVLON1 grown in absence of Trp were overpigmented compared with those of H26 (Supporting Information Fig. S1A). Interestingly, increasing the cellular concentration of NmLon by growing the cultures in presence of Trp produced a remarkable colourless phenotype in the cells. This indicates a modification in bacterioruberins content, the 50 carbon carotenoid pigments localized within the haloarchaeal membranes. To discard the possibility that this phenotype could be due to the presence of the heterologous protease and to further investigate the impact of Lon deficiency on the physiology *of H. volcanii*, we constructed a mutant strain deleted in the *lon* sequence that would express the homologous protease HvLon *in trans* (HVLON2). Taking into account the lethal phenotype of the *lon* mutation, we also constructed a conditional mutant (H26 $P_{tnaA}$-*lon-abi*) inserting the promoter P*tnaA* in the chromosome of *H. volcanii* upstream the *lon* gen (HVLON3). This allowed a tighter control of Lon protein synthesis. In addition, a deletion mutant in the downstream gene *abi* was also generated (HVABI) to evaluate the potential contribution of this protein to the Lon phenotypes. The mutant strains were verified by PCR as for HVLON1 (data not shown) and one clone of each was selected and used for further analyses. Fig. 3A shows a schematic representation of these mutants. To compare the endogenous Lon content in the parent H26 and mutant strains, cell lysates (cultures grown in Hv-Min without Trp) were probed by Western blotting with anti-NmLon antibodies (Fig. 3B). HVLON3 contained the lowest amount of Lon protein followed by HVLON2. When HVLON3 was complemented with the *wt* protein expressed from the P*tnaA* promoter of pTA963-*Hvlon*, the cellular Lon concentration in this strain was restored. On the other hand, Lon content remained unchanged in HVABI indicating that the absence of the downstream sequence had not affected the expression of the endogenous *lon* gene. Normal Lon levels were also restored when the mutant strains were grown in presence of Trp (0.1 mM) (data not shown).

### *Lon-deficient mutants evidence a growth defect and over-pigmentation*

Similarly to HVLON1, cultures of HVLON2 and HVLON3 evidenced increased pigmentation and reduced growth on Hv-Min agar plates without Trp (Supporting Information Fig. S1B). Examination of liquid cultures (Hv-Min without Trp) of the *wt* and mutant cells by phase contrast light microscopy revealed differences in cell shape (Fig. 3E). Cells of HVLON3 appeared pleomorphic, and many looked elongated compared with those of the *wt* strain. However, when the cultures were grown in presence of Trp (increasing Lon expression), this distinct phenotype was reversed. The HVABI mutant cells looked identical to the *wt* indicating that the effect on cell shape was due to a deficiency in Lon protein. To further evaluate the growth performance of these mutants, they were incubated in Hv-Min liquid medium in absence of Trp (Fig. 3C). Both *lon* mutants showed a defective growth phenotype

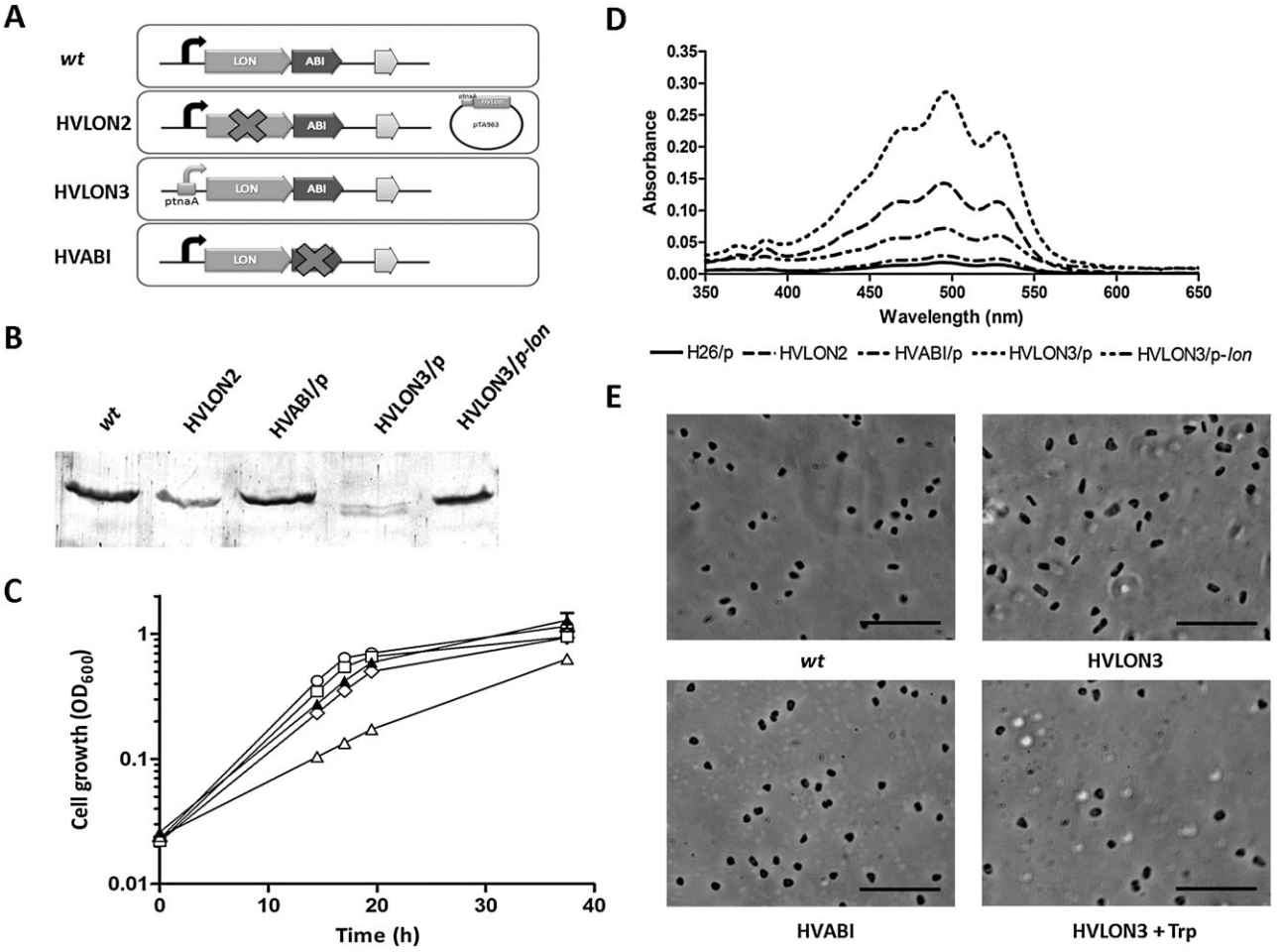


**Fig. 3.** Reduced growth and over-pigmentation of *lon* mutants.
A. Diagram of *H. volcanii lon* and *abi* mutants.
B. Western blot of cell lysates (prepared from the same number of cells) of *H. volcanii wt* and *lon* mutants (indicated in C) probed with anti-NmLon antibodies.
C. The *H. volcanii* strains H26/p (○), HVABI/p (□), HVLON3/p (Δ), HVLON3/p-*lon* (▲), HVLON2 (◊) were inoculated in Hv-Min medium from exponential phase cultures into 10 ml tubes ($OD_{600}$ 0.025), and growth was monitored by measuring $OD_{600}$; 'p': plasmid pTA963.
D. Absorption spectrum of acetone-soluble extracts of the *H. volcanii* strains harvested at $OD_{600}$ ~ 0.5. Extracts were prepared from the same number of cells. Experiments were performed in biological triplicate.
E. Phase contrast light microscopy of *lon* mutants (magnification 1000×). Size bar: 10 μm.

compared with H26, being the conditional mutant (HVLON3) the most severely affected ($0.204 \pm 0.010$ $h^{-1}$; $0.109 \pm 0.014$ and $0.028 \pm 0.002$ $h^{-1}$ for H26 *wt*, HVLON2 and HVLON3 respectively). This pattern correlated with the relative cellular concentration of Lon protein (23% and 9% relative to H26 *wt*). Growth rate was significantly improved in the HVLON3 when it was complemented *in trans* with the *wt Hvlon* gene (from $0.028 \pm 0.002$ to $0.133 \pm 0.004$ $h^{-1}$), while HVABI displayed normal growth ($0.183 \pm 0.014$ $h^{-1}$), demonstrating that the growth defect observed in HVLON3 was due to a suboptimal level of the Lon protease. Growth performance of HVLON2 and HVLON3 was also restored when these strains were grown in Hv-Min + Trp ($0.198 \pm 0.010$ $h^{-1}$).

To examine the effect of Lon deficiency on cell pigmentation, the cellular content of bacterioruberins was compared in all these strains in cultures grown to an $OD_{600}$ ~ 0.5. The spectrum of carotenoid pigments extracted from an equal amount of cells showed five distinct peaks at 370 nm, 389 nm, 475 nm, 496 nm and 530 nm corresponding to bacterioruberins (Fig. 3D). The concentration was ~ 8-fold and 14-fold higher in cells of HVLON2 and HVLON3 respectively, relative to that of H26 *wt* and HVABI. On the other hand, the complemented HVLON3 strain reduced bacterioruberins content from 14 to 4-fold. When the Lon-deficient mutants were induced to overexpress Lon, the cultures became completely colourless (data not shown).

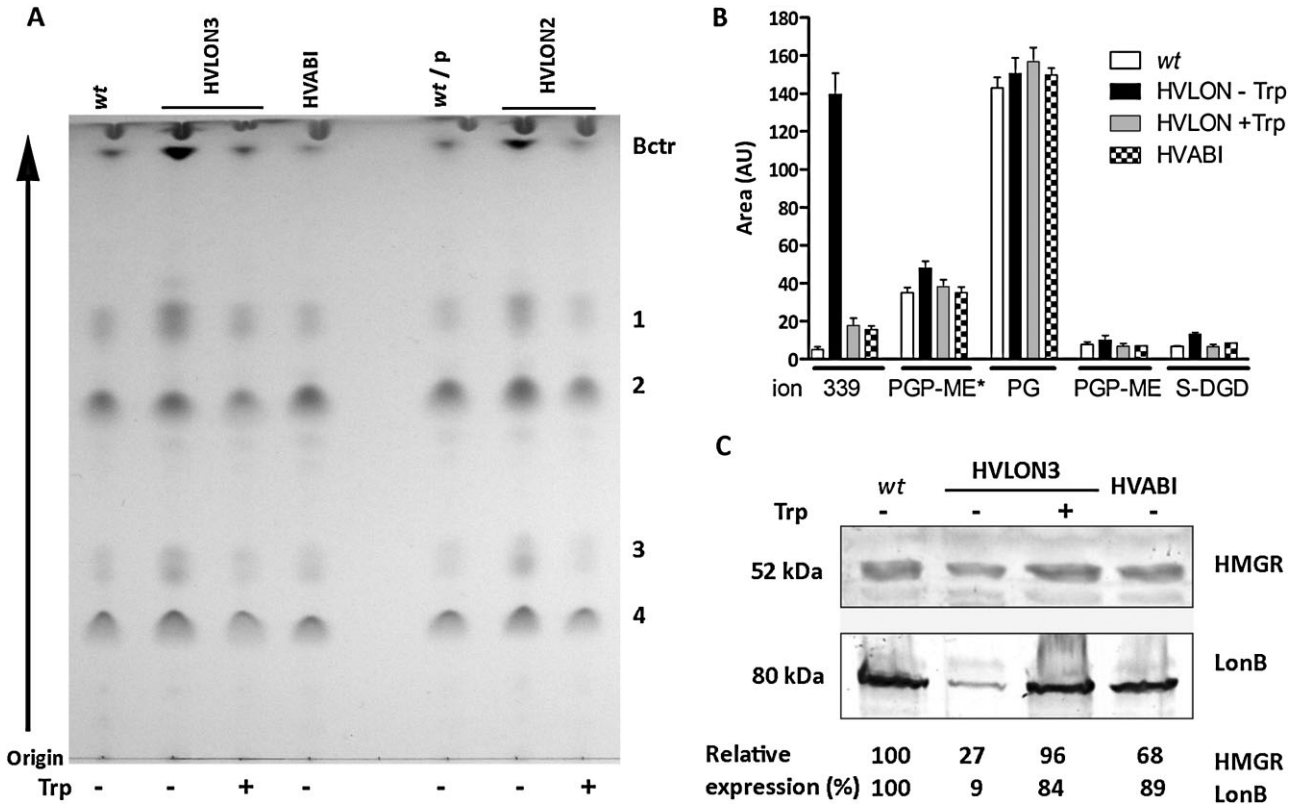


**Fig. 4.** Analysis of lipid content and composition in *H. volcanii* parent strain and the mutants deficient in Lon protease. Total lipids were extracted from *H. volcanii wt* and mutant strains grown in Hv-Min medium (in presence of Trp when indicated) ($OD_{600}$ ~ 0.7), using the Bligh-Dyer method.
A. Lipids were separated by TLC, and the spots were visualized with iodine vapours. Spots are named on the right. Bacterioruberins (Bctr). The results are representative of at least four independent experiments.
B. LC-ESI-MS/MS of total lipid extracts (Bligh-Dyer). The intensity (area) of the main peaks obtained by LC was quantified in the different strains and compared. Then, they were subjected to ESI-MS analysis. The experiment was performed in biological duplicates.
C. Analysis of HMGR by Western blotting. Cell lysates (prepared from the same number of cells) of the strains H26 *wt*, HVLON3 (+/– Trp) and HVABI were probed with rat anti-HMGR polyclonal antibodies (upper panel) and anti-NmLon antibodies (lower panel). The immunoreactive proteins were quantified using ImageJ software. Experiments were performed in biological duplicate and two different protein amounts were analysed.

### *Lon deficiency alters lipid content and composition in* H. volcanii

Taking into account the inverse correlation between the cellular Lon concentration and bacterioruberins content of the *lon* mutants, we hypothesized that Lon could be affecting (directly or indirectly) the isoprenoid biosynthetic pathway in *H. volcanii*. To test this possibility, the total lipid content of the Lon mutants and parent strain was examined by thin layer chromatography (TLC) analysis of Bligh-Dyer extracts (Fig. 4A). At least five distinct spots were consistently detected in all the strains which included neutral (Rf = 0.97) and polar lipids (Rf = 0.73–0.23). Within the first group, bacterioruberins content was significantly increased in the *lon* mutants, which agreed with our previous observation (Fig. 3D). Among the polar lipids (which were designated with numbers 1 to 4 based on their increased polarity) spot 1 showed the highest increase compared with the *wt* strain (3 and 5.7 fold in HVLON2 and HVLON3 respectively). Lipid 3 increased (2.5-fold) while the amounts of lipid 2 and 4 evidenced a slight increment (~ 1.4-fold). In presence of Trp, the lipid content of the *lon* mutants was restored, while HVABI did not evidence a differential phenotype. Although the different lipid species were not identified so far (except bacterioruberins), spot 4 corresponds to a glycolipid as it stained strongly with α-naphtol reagent and spots 2 and 3 to phospholipids based on $^{32}$Pi-Phospholipid labelling (Supporting Information Fig. S2). It was evident that *lon* mutants were affected in lipid content and composition.

To further investigate the effect of Lon deficiency on the cellular lipid content of *H. volcanii*, a preliminary analysis of lipid extracts by LC-ESI-MS was performed. The ESI-MS spectrum (negative ion) of the total cell lipid extracts of *H. volcanii* H26, HVLON3 –/+Trp and HVABI showed main diagnostic peaks (m/z values) at 449, 805,

899, 921 and 1055 which were assigned to phosphatidylglycerophosphate methyl ester (PGP-Me*) (bicharged peak), phosphatidylglycerol (PG), PGP-Me (monocharged peak), PGP-Me + Na and glycolipid monosulfated diglycosyl diphytanyl glycerol (S-DGD-1/S-DGD-5) respectively, based on comparison with m/z values of total lipids identified in *H. volcanii* (Corcelli and Lobasso, 2006) (Supporting Information Fig. S2). In addition, HVLON3 – Trp showed intense signals at m/z 311–325–339–353, which were not identified so far. Quantification of the areas corresponding to these peaks revealed a dramatic increase (27-fold) in these lipid species and to a lesser extent in S-DGD-1 (2-fold) in the cells with suboptimal Lon content (Fig. 4B). These concentrations returned to those of the *wt* strain when the mutant cells were induced to synthesize normal Lon levels (+ Trp). This result adds to the conclusion that lipid content is affected in the *lon* mutants. Considering the differences observed in lipid and bacterioruberin composition, we analysed the content of HMG-CoA reductase (HMGR), a key enzyme in the initial steps of the Mevalonate (MVA) pathway leading to isoprenoid biosynthesis in HVLON3 and control strains by Western blotting using human anti-HMGR polyclonal antibodies (Fig. 4C). An immunoreactive protein of the expected mobility for the *H. volcanii* HMGR (~ 52 kDa) (Bischoff and Rodwell, 1996) was detected in cell extracts of all the strains. A significant decrease in the intensity of this band (remaining 23% relative to H26 *wt*) was observed in HVLON3 in parallel with a reduced Lon content. When this strain was grown in presence of Trp and Lon expression was restored, HMGR levels also returned to normal. As for the phenotypes so far described, HVABI did not differ from the parent strain.

### *Lon-deficient strains show hypersensitivity to various antibiotics*

For further analysis of the *lon* phenotypes, the mutant strain HVLON2 was selected because it produced suboptimal amounts of Lon protein while still maintaining a growth performance fairly similar to that of the parent strain. We tested the impact of Lon deficiency on the sensitivity of *H. volcanii* cells to the antibiotics puromycin, novobiocin, lovastatin and bacitracin (Fig. 5). Growth was compared in the *wt*, HVLON2 and the complemented mutant strain (growth with Trp addition) in presence of different concentrations of these antibiotics.

Both the parent and HVLON2 strains were sensitive to puromycin, an inhibitor of translation in all cell types which generates abortive polypeptides, having very little to null growth at concentrations above 4 μM (Fig. 5A). The Lon-deficient cells were hypersensitive to puromycin and showed a severe decrease in growth at concentrations as low as 2 μM in contrast to the *wt* strain which was barely affected at this concentration. Cells induced to produce higher Lon concentrations (growth with Trp) recovered the same phenotype of the control.

Lovastatin is an inhibitor of HMGR (Atomi *et al*., 2012). Although growth was arrested in all strains at concentrations above 0.1 μM, the mutant cells were impaired to grow at lower lovastatin concentrations (0.05 μM) compared with the *wt* and complemented strains (Fig. 5B).

Bacitracin is a mixture of cyclic polypeptides that inhibits polar lipid and C50-carotenoid (bacterioruberins) synthesis in haloarchaea, probably by binding to polyisoprenyl pyrophosphates, precursor molecules in the biosynthetic pathway of isoprenoids (Moldoveanu and Kates, 1989). The Lon-deficient strain evidenced hypersensitivity to bacitracin (Fig. 5C). This mutant presented very little growth at concentrations above 100 μg ml$^{-1}$ relative to the *wt* strain which displayed minimal reduction in growth. Interestingly, HVLON2 showed increased resistance to bacitracin when it was grown in presence of Trp (condition that induces Lon expression).

Novobiocin inhibits gyrase and affects DNA replication (Atomi *et al*., 2012). We also tested the impact of this antibiotic at concentrations up to 0.5 μg ml$^{-1}$, however, all the strains were similarly affected.

The hypersensitivity of the *lon* mutants to puromycin, lovastatin and bacitracin does not seem to result from a non-specific effect caused by alterations in membrane permeability since HVLON2 cells were not differentially affected by novobiocin.

## Discussion

The biological role of the Lon protease has not been addressed in any archaeon yet. In this study, we show that LonB is essential for cell viability and growth in the model haloarchaeon *H. volcanii* (and probably in other archaea as well), since the only way of constructing a deletion mutant in the *lon* gene was by complementation with a copy of the *wt* gene (Fig. 2 and Fig. 3). Interestingly, the function of this protease seems to be conserved among haloarchaea with different lifestyles, as the lethal Δ*lon* phenotype observed in *H. volcanii* could be rescued introducing the *wt Nmlon* gene (Fig. 2). *Natrialba magadii* is extremely halophilic and strictly alkalophilic (3.5 M NaCl, pH 10) in contrast to *H. volcanii* which is moderately halophilic and neutrophilic (1.5–3.8 M NaCl, pH 7). Although LonA affects a number of cellular functions in bacteria and eukaryotic cells (Tsilibaris *et al*., 2006), the *lon* gene is not essential for cell viability, except in a few cases. In *Myxococcus xanthus,* which contains two copies of the *lon* gene, *lonV* is likely essential for viability, while *lonD* mutants are unable to sporulate (Tojo *et al*., 1993). In mammalian cells, mitochondrial Lon removes oxidatively

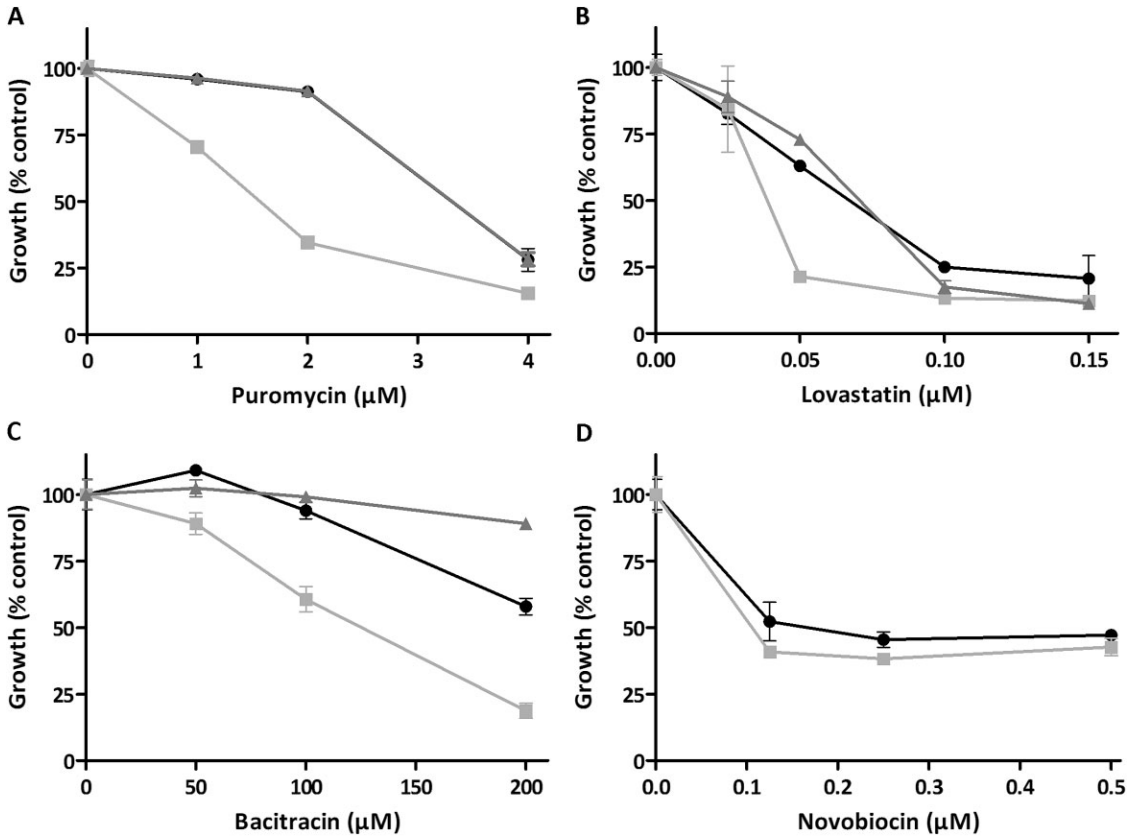


**Fig. 5.** Sensitivity of HVLON2 to different antibiotics. The H26 parent strain (●) and HVLON2 (–Trp ■; +Trp ▲) were grown in Hv-Min medium ($OD_{600}$ 0.1), then supplemented with different concentrations of antibiotic (puromycin, lovastatin, bacitracin or novobiocin) and incubated at 42°C for 20 h. Cell growth (%) was normalized to that observed in absence of antibiotic. Experiments were performed in biological triplicate.

damaged proteins, and those devoid of total Lon activity are induced to apoptosis (Bota *et al*., 2005).

LonB expression (transcripts and protein) was detected under different culture conditions in *H. volcanii* H26 *wt* (Fig. 1) and in *N. magadii* (Sastre *et al*., 2010), reflecting the importance of this enzyme in the physiology of haloarchaea. However, while changes in Lon mRNA content were observed after challenge with various stresses (Fig. 1), a correlation at the protein level was not evident. Similarly, a study on the proteasome of *H. volcanii* showed that while transcript levels changed as a consequence of transition from exponential to stationary phase, no significant differences were found in the protein content of several proteasome subunits (Reuter *et al*., 2004). It is likely that the LonB protease of *H. volcanii*, being an essential protein, maintains a fairly constant expression and the changes induced by the stress conditions at the transcript level affect the enzyme turnover with little impact on the total content of this protein. Additionally, post-translational modifications affecting the enzyme activity may be operating under changing physiological or environmental conditions.

Higher *lon* transcript expression was observed in cells that had been transferred from rich to minimal medium (Fig. 1A), suggesting that Lon is required in *H. volcanii* to cope with scarce nutrient availability, either to degrade endogenous protein resources and/or for the regulation of cellular functions necessary for adaptation to this condition. This observation is consistent with the function of Lon in bacteria as *E. coli lon* mutants are unable to adapt rapidly to a nutritional downshift (Kuroda *et al*., 2001). On the other hand, during amino acid starvation, the activation of the toxin-antitoxin (TA) HicAB system (which is conserved in free-living bacteria and archaea) was demonstrated to be Lon dependent (Jorgensen *et al*., 2009). Although the same culture conditions (shift from rich to minimum medium) could not be applied to test the phenotype of HVLON2 (P*tnaA* promotor is induced in rich medium containing Trp), upregulation of Lon expression (mRNA) in minimal medium (Fig. 1A) could account for the growth defect of Lon-deficient mutants growing in Hv-Min (Fig. 3C).

We observed a moderate (2-fold) but consistent decrease in the relative concentration of *lon* transcripts

in *H. volcanii* H26 growing in low salt medium (1.25 M NaCl) (Fig. 1B). Under this condition the cells became over-pigmented due to increased bacterioruberins concentration (2-fold). Similarly, an enhancement in pigmentation in low salt medium has been reported for *H. mediterranei* by D'Souza and colleagues (1997) who suggested that carotenoids could stabilize the membranes reducing cell lysis. According to our results, pigmentation of *H. volcanii* cells inversely correlated with the endogenous amount of Lon protein. The mutant strains HVLON2 and HVLON3, which synthesize suboptimal levels of Lon protease, had higher bacterioruberins content than the parent strain (Fig. 3D). This effect was more striking (14-fold) in the conditional mutant (HVLON3) which expresses the lowest level of Lon protease. When the mutant strains were complemented *in trans* with Lon protein or grown in the presence of Trp, they recovered the normal pigmentation. Moreover, over-expression of Lon rendered the cultures completely colourless. These findings agree with the results obtained for the *wt* strain H26 growing in non-optimal low salt conditions in which overpigmented cells displayed a lower Lon (mRNA) expression. Bidle and colleagues (2007) reported that bacterioruberins and total lipid content decreased in *H. volcanii* cells exposed to non-optimal high salt medium (3.5 M NaCl), observation that could be correlated with elevated Lon expression. When the mutant strain HVLON2 was challenged to grow in Hv-Min containing various salt concentrations (1.25 M, 2.5 M and 3.8 M NaCl), it showed a similar performance as the parent strain except in 3.8 M NaCl, condition in which the cells evidenced a modest defect in growth rate (data not shown). It is possible that the basal amount of Lon protein expressed in the mutant cells allowed them to cope with salt stress. Alternatively, the proteasome could compensate for the Lon deficiency under this condition as it has been reported that this protease protects the cells of *H. volcanii* and *Halobacterium* from low salt stress (Zhou *et al*., 2008; Chamieh *et al*., 2012).

*Haloferax volcanii* cells irradiated with UV light diminished the level of *lon* mRNA (Fig. 1C). Taking into account that carotenoids represent a photoprotective mechanism against UV damage in various types of organisms, including haloarchaea (Shahmohammadi *et al*., 1998), a decrease in Lon expression would be expected to provide the cells with a suitable content of bacterioruberins that would protect them from exposure to UV light.

Carotenoid metabolism in haloarchaea seems to be somehow regulated by proteolysis since accumulation of these compounds was found under conditions of Lon deficiency (this work), while white colonies were produced in some proteasome mutants in *H. volcanii* (Humbard *et al*., 2010).

Lon-deficient mutants not only displayed a notorious increase in carotenoids but also changed their content in some polar lipids, as evidenced by analysis of Bligh-Dyer extracts using TLC and LC-ESI-MS (Fig. 4, Supporting Information Fig. S2). While some of these lipids were not identified in this study, it is worth noting that the relative intensity of two spots was enhanced three to six-fold, and the species with m/z ranging from 311 to 353 showed a very remarkable increment (27-fold) in the HVLON3 mutant. A more detailed analysis of these lipids will be performed in future studies. The membrane lipids of archaea are polyisoprenoid compounds which are synthesized by means of a modified MVA pathway (Matsumi *et al*., 2011). A key enzyme acting in the first steps of this route is HMGR, which catalyses the conversion of two molecules of acetyl-CoA to mevalonate. The elevated levels of carotenoids and polar lipids observed in the *lon* mutants could be due to increased amounts of HMGR protein as a consequence of its stabilization in the Lon protease-deficient strains. To test this hypothesis, we compared the content of this enzyme in the mutant and *wt* strains by Western blotting. Unexpectedly, the cellular content of HMGR was reduced in the mutant relative to the *wt*, suggesting that instead of being a Lon substrate, HMGR could be downregulated as a consequence of accumulation of the end products of the MVA pathway. The *lon* mutant was also hypersensitive to lovastatin and bacitracin (Fig. 5), behaviour that would be expected if decreased levels of HMGR and the precursor molecules isoprenyl pyrophosphates were present in the *lon* mutants respectively. Surprisingly, the mutant strain growing in presence of Trp which over-expressed Lon protein was even more resistant to bacitracin than the *wt* cells. This observation would make sense with a reduced level of end products and a positive feedback to the biosynthetic pathway in cells containing a higher Lon content. In *H. volcanii*, cell membrane biosynthesis is regulated in response to changes in medium salinity through modifications of the MVA pathway (Bidle *et al*., 2007). This haloarchaeon increased HMGR expression under non-optimal high salt conditions (3.5 M NaCl) to compensate for the decrease in carotenoids and membrane lipids. In this context, we propose that the LonB protease is involved in the regulatory mechanism that controls membrane lipid composition in *H. volcanii*. A more thorough analysis is required to ascertain what the exact effect of the Lon protease is in the isoprenoid biosynthetic pathway of haloarchaea. The clear deregulation of carotenoid and lipid biosynthesis could explain why the Lon protease is essential for *H. volcanii* physiology since the accurate regulation of lipid turnover is of fundamental importance to maintain the integrity and functionality of the cell membrane, particularly in microorganisms thriving in extreme habitats.

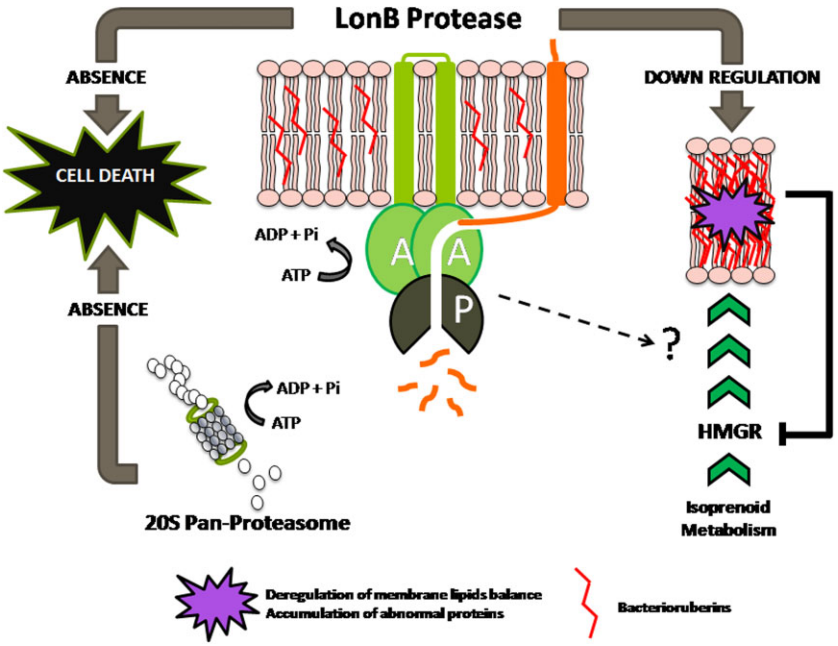


**Fig. 6.** Schematic representation of the enzymes involved in ATP-dependent proteolysis in *H. volcanii* with emphasis in Lon function. The 20S PAN-proteasome (soluble enzyme complex) degrades cytosolic misfolded proteins generated during thermal and hypo-osmotic stress (Zhou *et al*., 2008), while the membrane-bound LonB protease removes abnormal proteins most likely membrane associated. The biological functions of these proteases are not redundant (at least not completely) since absence of each protease renders *H. volcanii* cells unviable. Downregulation of LonB expression induces changes in membrane lipids balance which likely affects cell survival (this work).

The Lon-deficient mutant was hypersensitive to puromycin showing that the archaeal enzyme is also involved in protein quality control, a function that is conserved in Lon proteases.

The inability of *H. volcanii* Δ*lon* mutant cells to survive is likely a consequence of the absence of other enzyme/s able to degrade Lon substrates. Archaeal genomes encode only two ATP-dependent proteases: the proteasome (a soluble enzyme) and a membrane-bound LonB type protease. In *H. volcanii* the archaeal proteasome degrades miss-folded proteins generated during thermal and hypo-osmotic stress, and the core 20S components are essential for growth (Zhou *et al*., 2008). Based on this evidence and the data presented in this work, we propose that the complementary functions of the 20S proteasome and LonB are needed for the ATP-dependent proteolysis in archaeal cells. The metalloprotease FtsH is the only essential and membrane-associated ATP-dependent protease in *E. coli*. This protease controls several important physiological processes; however, the essential function of FtsH in *E. coli* is due to its role in the regulation of the biosynthetic pathway of membrane lipids such as the lipopolysaccharide (Langklotz *et al*., 2012). As archaea lack the FtsH protease, it has been suggested that the archaeal-type Lon could be a functional homologue of FtsH. Fig. 6 shows a schematic representation of the enzymes involved in ATP-dependent proteolysis in *H. volcanii* with emphasis in Lon function.

We have recently started a proteomic analysis of the *lon* mutants to identify potential targets of this protease. The outcomes of this research will help to clarify the role of the archaeal LonB in the metabolism of isoprenoids as well as to discover the cellular processes affected by this universally conserved ATP-dependent protease in archaea.

## Experimental procedures

### *Strains and culture conditions*

Microorganisms used in this study are indicated in Table 1. *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium supplemented with ampicillin (100 μg ml$^{-1}$) or kanamycin (50 μg ml$^{-1}$) when needed. *Haloferax volcanii* strains were grown at 42°C in Modified Growth Medium (MGM) or minimal medium (Hv-Min). Medium formulae were according to *The Halohandbook* (Dyall-Smith, 2008). Liquid cultures were aerated at 200 r.p.m., and solid cultures were grown on 1.5% agar plates. Cell growth was monitored by measuring the optical density of the cultures at 600 nm ($OD_{600}$). *Escherichia coli* DH5α was used for routine cloning and transformed by the $CaCl_2$ method (Sambrook and Russell, 2001).

Haloarchaeal media were supplemented with novobiocin (2 mg ml$^{-1}$), 5-fluoroorotic acid (5-FOA) (50 μg ml$^{-1}$) and uracil (10 μg ml$^{-1}$ and 50 μg ml$^{-1}$ for growth in the presence and absence of 5-FOA respectively) as needed. For nutritional stress, *H. volcanii* cultures were grown in MGM medium ($OD_{600}$ 0.4), centrifuged under sterile conditions, and the cells were suspended in fresh MGM or Hv-Min medium. The cultures were further incubated and sampled. For osmotic stress studies, *H. volcanii* cultures growing in MGM medium (2.5 M NaCl, $OD_{600}$ 0.7) were diluted with fresh medium ($OD_{600}$ 0.2) to final NaCl concentrations of 1.25 M, 2.5 M and 3.8 M. The cultures were further incubated and sampled. For UV stress, *H. volcanii* cultures ($OD_{600}$ 0.2–0.3) were transferred to 9 cm sterile Petri dishes (10 ml/plate), placed on a rotatory shaker (50 r.p.m.) and irradiated with UV-B tubes (TL100W/12, Phillips, Eindhoven, Netherlands) for 8 min (15 J m$^{-2}$) at 18°C. After irradiation, the cells were

**Table 1.** List of strains, plasmids and primers used in this study.

| Strains and plasmids | Phenotype, genotype or oligonucleotides sequences | Source/reference |
|---|---|---|
| Strains | | |
| *Escherichia coli* strains | | |
| DH5 | F– *recA1 endA1 gyrA96 thi-1 hsdR17*($r_k$–$m_k^+$) *supE44 relA1 lac* [F′ *proAB lacI*$^q$Z ΔM15::Tn*10*(Tet$^r$)] | NE BioLabs |
| *OneShot*® TOP-10 | F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*Z ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str$^R$) *end*A1 *nup*G λ- | Invitrogen |
| GM33 | LAM-, IN(rrnD-rrnE)1, F– *dam-3 sup-85* (Am) | Marinus and Morris (1974) |
| *Haloferax volcanii* strains | | |
| H26 | Δ*pyrE2* derivative of DS70 | Allers and colleagues (2004) |
| HVLON1 | H26 Δ*lon* pRV1-*Nmlon* (devoid of HvLon, low expression of NmLon in absence of Trp) | This study |
| HVLON2 | H26 Δ*lon* pTA963-*Hvlon* (devoid of HvLon, low expression of HvLon in absence of Trp) | This study |
| HVLON3 | H26 $P_{tnaA}$-*lon-abi* (low expression of Lon and Abi in absence of Trp) | This study |
| HVABI | H26 Δ*abi* (devoid of Abi) | This study |
| Plasmids | | |
| pTA131 | Ap$^R$, integrative vector based on pBluescript II, $P_{fdx}$-*pyrE2* marker, | Allers and colleagues (2004) |
| pTA13-*lonupdw* | pTA131 with 700 bp of 5′ and 3′ flanking regions of *Hvlon* | This study |
| pTA13-*abiupdw* | pTA131 with 700 bp of 5′ and 3′ flanking regions of *Hvabi* | This study |
| pRV1 | Ap$^R$, Nov$^R$, $P_{tnaA}$ promoter, promoter-less *bgaH* | M. Dyall-Smith, pers. comm. |
| pRV1-*Nmlon* | pRV1 containing $P_{tnaA}$-*Nmlon* | Sastre and colleagues (2010) |
| pTA963 | Ap$^R$, over-expression vector, $P_{tnaA}$ promoter, N-terminal 6XHis tag, *pyrE2* and *hdrB* markers, pHV2 origin. | Allers and colleagues (2004) |
| pTA963-*Hvlon* | pTA963 containing $P_{tnaA}$-*Hvlon* | This study |
| pTA963-*Hvabi* | pTA963 containing $P_{tnaA}$-*Hvabi* | This study |
| Primers | | |
| iPCR-R | 5′-AAGGAGATGGTCGACATCAT-3′ | This study |
| 7S-F | 5′-GATAGGTCGGGCAGTTTGG-3′ | Paggi and colleagues (2010) |
| 7S-R | 5′-AATCCCGGTTGCCTTCTC-3′ | Paggi and colleagues (2010) |
| fwuplonhfx | 5′-tcaagcttTCGACCACGACGAGATGGAC-3′ | This study |
| rvuplonhfx | 5′-tgaattcAGAACTGTCGTCGCTACTTCT-3′ | This study |
| rvdwlonhfx | 5′-atctagaACGATGATGCCGAGTCGA-3′ | This study |
| LonHfxverif5 | 5′-GTTGTCGGGACCGACGAAGAC-3′ | This study |
| HvlonNdeI-F | 5′-gtatcatATGAGTAACGATACGAACAC-3′ | This study |
| HvlonBamHI-R | 5′-gtggatccTTACTGCGGGCTGGGGCTGGAG-3′ | This study |
| ptnaAHvlon-F | 5′-ggatccGCCCGTTCTCGTCGCGCTC-3′ | This study |
| ptnaAHvlon-R | 5′-tctagaTTACTGCGGGCTGGGGCTGG-3′ | This study |
| fwupabihfx | 5′-*ctcgag*CGCGGACCAGTACATCGAG-3′ | This study |
| rvupabihfx | 5′-*aagctt*CCGGCGTTACTGCGGG-3′ | This study |
| fwdwabihfx | 5′-*ggatcc*CGGGGTCTCCCTGCGTC-3′ | This study |
| rwdwabihfx | 5′-*tctaga*ACTGACCGTCCCGACCGACC-3′ | This study |

transferred to the original flasks, left to recuperate for 1 h at 42°C (250 r.p.m.) and sampled. Under all conditions, the samples were used to measure cell growth ($OD_{600}$), Lon mRNA and protein content by Northern and Western blotting respectively. Growth rate (μ, $h^{-1}$) was calculated from the slope of the growth curve × 2.3.

### *RNA isolation and Northern blotting analysis*

Total RNA was isolated from cell pellets of *H. volcanii* H26 as described previously (Nieuwlandt *et al*., 1995). Contaminant proteins and DNA were eliminated by phenol/chloroform/isoamylic alcohol (25:24:1) extraction and DNaseI treatment respectively. Ribonucleic acid samples (15 μg per lane) were suspended in glyoxal sample buffer electrophoresed and transferred to a positively charged nylon membrane (Hybond-N+) (Amersham Biosciences, Piscataway, NJ, USA) by capillary blotting DNA probes (Lon and 7S RNA) amplified by PCR, were labelled with [α-$^{32}$P] dCTP using a random DNA labelling kit. Membranes were pre-hybridized with Church buffer (1% BSA, 1 mM EDTA, 0.5 M phosphate buffer pH 7.2, 7% SDS) each probe (denatured at 100°C for 5 min) was added to the membranes and further incubated at 65°C for 16 h. Membranes were washed at high stringency (up to 0.1X SSC–0.1% SDS at 65°C) and incubated 10–60 min on imaging plates (Fuji) at room temperature, visualized with a Storm analyser (Amersham Biosciences) and the signals quantified using ImageQuant software (Molecular Dynamics, Sunny vale, CA, USA). Lon expression was normalized relative to that of the constitutively expressed 7S RNA.

### *Chromosomal knockouts of* lon *and* abi *genes*

The *lon* (HVO_0783) and *abi* (HVO_0784) genes were targeted for deletion from the chromosome of *H. volcanii* H26 by the *pyrE2*-based pop-in pop-out method (Allers *et al*., 2004). Briefly, primer pairs (uplonhfx/dwlonhfx for *lon* and upabihfx/dwabihfx for *abi*) (Table 1) were designed to amplify ~ 700 bp of genomic DNA flanking the 5′- and 3′- ends of the coding region of each target gene using high-fidelity IProof

DNA polymerase. The PCR-generated DNA fragments were cloned sequentially into the suicide plasmid pTA131 using NdeI/BamHI and NdeI/EcoRI restriction sites for *lon* and *abi* respectively. Plasmids were sequenced to confirm fidelity and used as templates for homologous recombination with the *H. volcanii* genome. Transformants were screened for integration in the absence of uracil (Hv-Ca ura$^-$) and then for excision of the plasmid in 5-FOA. Deletion of the respective genes from the chromosome was confirmed by PCR using primers specific for the coding region (HvlonNdeI-F and HvlonBamHI-R) and primers external to the 5′ and 3′ flanking regions (rvdwlonhfx and LonHfxverif5) and by dot blotting (absence of *lon* sequence).

### *P*tnaA *promoter fusion to the* lon *gene*

The tryptophan-regulated promoter (P*tnaA*) was inserted upstream the *lon* gene in *H. volcanii* H26 chromosome. The region of 2.3 kb P*tnaA–Hvlon* from the pTA963-*Hvlon* plasmid was amplified using primers ptnaAHvlon-F and ptnaAHvlon-R (Table 1) and inserted downstream the pTA131*Hvup* vector using BamHI and XbaI restriction sites. This plasmid was used to insert the P*tnaA* promoter upstream of *lon* via the pop in-pop out method. Colonies were screened by patching onto Hv-Min plates with and without 2 mM tryptophan (Trp). Colonies that looked over-pigmented and deficient in growth in the absence of Trp were confirmed to have incorporated the P*tnaA* promoter analyzing the conditional expression of Lon protein in absence/presence of Trp by Western blotting.

### *Western blotting*

*H. volcanii* cultures ($OD_{600}$ 0.6–0.9) were harvested by centrifugation, cell pellets were suspended in SDS-PAGE sample buffer ($OD_{600}$ 25), and proteins were fractionated by SDS-PAGE (8% acrylamide gels) and stained with Colloidal Coomasie Blue (Candiano *et al*., 2004). A replica gel was transferred to a nitrocellulose membrane, and probed with the corresponding antibodies: rabbit polyclonal anti-NmLon (1/10 000) (Sastre *et al*., 2010) or anti-HMGR [HMGCR (H-300) rabbit polyclonal antibody raised against amino acids 589–888 mapping at the C-terminus of HMGCR of human origin, Santa Cruz Biotechnology] for 16 h at 4°C. Blots were washed, incubated with alkaline phosphatase-conjugated secondary anti-body (1/10 000) (Amersham) for 2.5 h and developed with 5-bromo-4-chloro-3-indolyl phosphate/p-nitroblue tetrazolium (BCIP/NBT). The intensity of the protein bands was quantified using ImageJ Software (National Institutes of Health, Bethesda, Maryland; http://rsbweb.nih.gov/ij/). Full-Range Rainbow Molecular Weight Markers (Amersham) were used as molecular mass standards.

### *Lipid extraction and analysis*

Total lipids were extracted from *H. volcanii* cultures ($OD_{600}$ ~ 0.7; 25 ml) using the Bligh-Dyer method with chloroform/methanol/water according to Bidle and colleagues (2007). Lipid extracts were suspended in chloroform and analysed by TLC on 20 × 20 cm, 0.25 mm Silica gel 60 glass plates (Sigma-Aldrich) using chloroform/methanol/acetic acid/water (85:22.5:10:4) (Sprott *et al*., 2003). Spots were detected using iodine vapour and were quantified using ImageJ Software. For electrospray ionization-mass spectrometry (ESI-MS) analysis, lipid extracts were evaporated under $N_2$ stream. Negative ion ESI-MS analysis was performed using a Bruker micrOTOF-Q II mass spectrometer (Bruker Daltonics, Freemon, CA, USA) equipped with an electrospray ionization source. Analyses were carried out in the loop injection mode with dried total lipid extracts dissolved in chloroform/methanol (1:4). Samples injected via a 10 µl loop were transferred to an MS electrospray interface (ESI) at flow rate of 10 µl min$^{-1}$. The experiments were performed in biological duplicate.

### *Bacterioruberin determination*

Cultures were grown in Hv-Min medium ($OD_{600}$ 0.5). Equal amounts of cells ($3 \times 10^8$ CFU ml$^{-1}$) were suspended in acetone/methanol (1:1), incubated 1 h at room temperature, centrifuged and the supernatant recovered. Bacterioruberin concentration was measured spectrophotometrically at absorbance 350–650 nm in a GeneQuant 1300 spectrophotometer (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The absorbance at 496 nm (highest peak) was used to compare the bacterioruberin content of the different samples.

### *Antibiotic treatment*

Cultures of H26 and HVLON2 were grown in Hv-Min (-ura) ($OD_{600}$ ~ 0.1) and then supplemented with different concentrations of puromycin (0, 1, 2, 4 µM), lovastatin (0, 0.025, 0.05, 0.1, 0.15 µM), bacitracin (0, 50, 100, 200 µg ml$^{-1}$), and novobiocin (0, 0.125, 0.25, 0.5 µg ml$^{-1}$). Growth ($OD_{600}$) was measured after 20 h.

### *Light microscopy*

The *H. volcanii* H26 *wt*, HVLON3 and HVABI strains were grown in liquid Hv-Min cultures (HVLON3 with and without Trp) to an $OD_{600}$ ~ 0.3. The cultures were centrifuged and suspended in Hv-Min medium to an $OD_{600}$ ~ 1. The cells were loaded onto glass slides and observed under the light microscope (phase contrast) using an Eclipse Ti-S inverted microscope (NIKON).

## Acknowledgements

This work was supported by grants from CONICET (PIP 1783) and UNMDP, Argentina. M. C. is a PhD student and research fellow from CONICET, Argentina. We thank Dr M. Garcia de Bravo (UNIBIOLP, UNLP-CONICET) for providing HMGCR (H300) rabbit polyclonal antibody; Dr A. M. Laxalt and Lic. J. M. D' Ambrossio (IIB-CONICET-UNMDP) for assistance in lipid analysis; Bioq. Gustavo Millán (CCT-CONICET, Rosario Argentina) for assistance in the LC-ESI-MS analysis.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Fig. S1.** Differential pigmentation and growth of *H. volcanii* mutant strain deficient in Lon protease. A. The parent (H26) and HVLON1 mutant strains were cultured in Hv-Min medium in absence and presence of Trp (2 mM) and harvested at $OD_{600}$ 1.5. B. The parent (H26) strain transformed with empty plasmid pTA963 and mutant strains HVLON2 and HVLON3 were grown on solid Hv-Min medium in absence of Trp.

**Fig. S2.** Identification of glycolipids and phospholipids in *H. volcanii* H26 *wt*. A–B. Total lipids were extracted from *H. volcanii* H26 *wt* cells and subjected to TLC analysis as described in Experimental Procedures. Total lipids were visualized with iodine vapours and then, the TLC plate was sprayed with α-naphtol reagent (2.4% α-naphtol, 10% sulphuric acid, 80% ethanol) and heated at 120°C 3–5 min to identify glycolipids (pink-purple spots). C. $^{32}$P-Phospholipid labelling and TLC analysis. *Haloferax volcanii* cells suspended in HvMin medium (200 μl) were incubated with 5 μCi carrier-free $^{32}PO_4^{3-}$ (Amersham Biosciences) for 3 h and the reaction stopped by adding 20 μl of 50% perchloric acid. Total lipids were extracted as described and analysed by TLC. $^{32}$P-Phospholipids were visualized using a Storm image analyser. Spot 4 was identified as a glycolipid while spot 2 and 3 as phospholipids.

**Fig. S3.** Electrospray ionization-mass spectrometry (ESI-MS) analysis of total lipids from *H. volcanii* H26 *wt* and Lon mutant strains. Total lipids extracted from *wt*, HVLON3 (grown without and with Trp), and HVABI were dissolved in chloroform/methanol (1:4) and subjected to ESI-MS analysis (negative ion). Main diagnostic peaks (m/z values) at 449, 805, 899–921 and 1055 were assigned to phosphatidylglycerolphosphate methyl ester (PGP-Me) (bicharged peak), phosphatidylglycerol (PG), PGP-Me (monocharged peak) and glycolipid monosulphated diglycosyl diphytanyl glycerol (S-DGD-1/S-DGD-5) based on comparison with m/z values of total lipids identified in *H. volcanii* (Corcelli and Lobasso, 2006). The bar on top of 325–339 ions denotes the lipids that were increased in content in the strain deficient in Lon protease.

**Table S1.** List of strains, plasmids and primers used in this study.